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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/743,195	03/01/2001	Christopher J. Lloyd	39-228	7373

23117 7590 03/29/2005

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EXAMINER	
HAQ, SHAFIQUL	
ART UNIT	**PAPER NUMBER**
1641	

DATE MAILED: 03/29/2005

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)
	09/743,195	LLOYD ET AL.
	Examiner	**Art Unit**
	Shafiqul Haq	1641

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) 1-29 is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) 1-29 is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☒ The specification is objected to by the Examiner.

10)☒ The drawing(s) filed on 01 March 2001 is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☒ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☒ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☒ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)

2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)

3) ☒ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date 7/10/2001,1/8/2001.

4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.

5) ☐ Notice of Informal Patent Application (PTO-152)

6) ☐ Other: _____.

DETAILED ACTION

Claim Objections

1. The title of the invention is not descriptive. A new title is required that is clearly indicative of the invention to which the claims are directed. The following title is suggested: "Analysis of a sample using its characteristic cycle time."
2. This application does not contain an abstract of the disclosure as required by 37 CFR 1.72(b). An abstract on a separate sheet is required.

Claim Rejections - 35 USC § 112

The following is a quotation of the second paragraph of 35 U.S.C. 112:

The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 1-9, 13, 24 and 27 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

3. Claim 1 is vague and indefinite as it is unclear what is meant by the terms "characteristic cycle time" and "active elements" in lines 1-2.
4. Claim 1 is vague and indefinite. The term "some" in line 3 is a relative terminology which fails to define the number of active elements in the sample.

5. The phrase "correlating the detected signal with itself" renders claim 2 indefinite because it is unclear what is meant by this phrase. Detected signal is correlating with what?
6. Claim 3 is vague and indefinite because it is unclear what is "a suitable modification of the active element's environment". It is also unclear what is meant by the term "active element's environment".
7. Claim 4 recites the term "the modification of the active elements' environment". There is insufficient antecedent basis for this limitation in the claim.
8. Claim 4 in indefinite. The term "some" in line 1 fails to define the number of active elements in the sample. It is unclear what is meant by the term "lifetime". The term "this" in line 8 also renders the claim indefinite because it is unclear what does this refer to?
9. Claim 5 is indefinite because the term "may be" in line 5 is not a positive recitation.
10. The term "able to" in line 3 of claim 6 renders the claim indefinite because the term "able to" is not a positive recitation.
11. The term "modifying moiety" in claims 6-8 & 10 renders the claims indefinite, as it is not clear what is encompassed by the term.
12. Claim 7 recites the limitation "the change in cycle time" in line 1. There is insufficient antecedent basis for this limitation in the claim.
13. Claim 8 recites the limitation "the conformation" in line 2. There is insufficient antecedent basis for this limitation in the claim.

14. Claim 9, part (e) is vague and indefinite because it is unclear what is meant by altering "the overall" size of the probe.

15. Claim 13 recites the limitation "quenching moiety" in line 3. There is insufficient antecedent basis in the claim.

16. Claim 24 recites the limitation "excitation cross section of a ground state" in line 1. There is insufficient antecedent basis for this limitation in the claim. The term "excitation cross section of a ground state" is not mentioned in claim 1 which it referred to. The above term makes the claim indefinite and thus appropriate correction is required.

17. Claim 27 depends on claim 1 and recites the limitation "excitation of the sample" in line 1. There is insufficient antecedent basis for this limitation in the claim. In claim 1, line 2, "exciting active elements in the sample" is referred to. Appropriate correction is required.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

18. Claims 1-4, 26 and 29 are rejected under 35 U.S.C. 102(b) as being anticipated by Soper (Soper et al., Detection and identification of single molecules in solution, J. Opt. Soc. Am. B, 1992, Vol 9, No 10, 1761-1769).

Soper discloses a method and apparatus for detection of single molecules in a solution including the following : (i) excitation means for excitation of active elements (fluorescent species) in a sample (page 1761, right column, lines 22-25, 39-41 & page 1762, left column, lines 1-2 and Fig.1) (ii) detecting individual quanta in a stream (page 1765, right column, lines 7-9; page 1767, right column, lines 8-11, 28-39 and figs. 10 & 11) (iii) correlation of detected signal (page 1765, right column, lines 7-11). (iv) determination of characteristic cycle time (page 1767, right column, lines 8-11, 28-39; page 1768, right column, lines 1-4, 8-12) and (v) suitable modification of active element environment(page 1766, right column, lines 6-9).

"Characteristic cycle time" reads on characteristic "fluorescence lifetime" since in lines 29-30 of the specification, "Characteristic cycle time" is defined as the time taken for an active element to return to a ground state following excitation to and excited state.

Upper level of emission transition, lower level of emission transition and emission of detectable quanta upon relaxation are inherent properties of fluorescent species in fluorescence emission and are not given any patentable weight.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

19. Claims 3-28 are rejected under 35 U.S.C. 103(a) as being unpatentable over Soper in view of each of 1) Lakowicz (US patent # 5631169) and Walt (Walt et al, US patent # 5254477).

Soper et al. teach a method for detection of characteristic cycle time including detection of individual quanta in a solution as discussed above, However, Soper et al. fails to teach collisional quenching, spacer arm and other modifications as claimed in claims 5-27.

Lakowicz discloses photoluminescent energy transfer (FRET) between donor and acceptor (fluorescent resonant energy transfer species) to measure biological reaction.

As for claims 5-8 and10-13, Lakowicz discloses using various sandwitch-type assays (eg. ELISA) which includes active elements bound to a substrate (column 2, lines 37-43, 48-51). Lakowitz also discloses use of quenches (modifying moiety) and collisional quenching (column 2, lines 30-37) to measure change in fluorescence lifetime due to transfer of energy to quencher (modifying

moiety) and vibrational motion is encompassed in collisional quenching mechanism.

As for calims 22-25 and 27 Lakowicz discloses use of fluorescence resonance energy transfer species (column 16, lines 35-50 and claim 1) to monitor immune reaction in solution (see abstract) and excitation of the sample by chemical reaction (column 1 lines 48-54).

As for claims 9 and 14-21, Lakowicz discloses that the efficiency of energy transfer depends on relative distance and orientation between the donor and the acceptor (column 3, lines 36-40). Lakowicz further indicates that modification can be made within the scope of the invention (column 18, lines 9-11) such as the choice of photo luminescent energy transfer donor and acceptor such that the donor and acceptor are brought to close proximity when the binding partners bind each other (see claim 1).

Lakowicz fails to disclose the use and modification of linker or spacer arm.

Walt in a method requiring fluorescence intramolecular energy transfer discloses use of spacer or linker arm and modifcation/change of the length and flexibility of spacer arm for detection of an analyte in a sample (Column 9, lines 19-24 and 50-68).

Optimization of the length and sequence of the linker between the donor and acceptor are commonly done for each specific application as in many cases, steric hindrance or folding interference can occur between the donor and acceptor if the linker is not sufficiently long and flexible.

Therefore, it would have been obvious at the time of the invention to a person of ordinary skill in the art to introduce spacer arm as taught by Walt in the immunoassay method of Lakowicz and use Soper's method to derive characteristic lifetime (characteristic cycle time) change due to FRET as a measure to detect biological reaction.

20. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Shafiqul Haq whose telephone number is 571-272-6103. The examiner can normally be reached on 7:30AM-4:00PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Long V. Le can be reached on 571-272-0823. The fax phone number for the organization where this application or proceeding is assigned is 703-872-9306.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Shafiqul Haq
Shafiqul Haq
Patent Examiner, Art Unit 1641

Long Le
LONG V. LE
SUPERVISORY PATENT EXAMINER
TECHNOLOGY CENTER 1600
03/21/05

Sheet 1 of [redacted]

INFORMATION DISCLOSURE CITATION

(Use several sheets if necessary)

ATTY. DOCKET NO.	SERIAL NO.
39-228	09/743,195
APPLICANT	
LLOYD et al.	
FILING DATE	**GROUP**
January 8, 2001	

U.S. PATENT DOCUMENTS

*EXAMINER INITIAL		DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE
SH		4421860	12/83	Elings et al			
		5302349	4/94	Dandliker et al			
		5090818	2/92	Kleinerman			

FOREIGN PATENT DOCUMENTS

		DOCUMENT	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION YES	NO
SH		WO 96/27798	9/96	WIPO				
		WO 92/13265	8/92	WIPO				
		WO 92/19957	11/92	WIPO				
		WO 96/27798	9/96	WIPO				
		WO 94/18218	8/94	WIPO				
		0713089 A2	5/96	European				
		0552107 A1	7/93	European				
		0552108 A3	7/93	European				
		0552108 A2	7/93	European				
		0556509 A2	8/93	European				
		0561653 A1	9/93	European				
		2284667A	6/95	Great Britain				

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent pages, etc.)

SH	PATENT ABSTRACTS OF JAPAN, Vol. 011, No. 137 (P-572), 2 May 1987 & JP 61 277060A (JOKO:KK), 8 December 1986

*Examiner	[signature]	Date Considered	1/28/05

Examiner: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to application.

Form PTO-FB-A820 (Also PTO-1449)

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INFORMATION DISCLOSURE CITATION

(Use several sheets if necessary)

ATTY. DOCKET NO. 39-228	SERIAL NO. Unknown 09/743195
APPLICANT LLOYD et al.	
FILING DATE January 8, 2001	GROUP

U.S. PATENT DOCUMENTS

*EXAMINER INITIAL		DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE
SH		4,421,860	12/1983	ELINGS VIRGIL B et al.			

FOREIGN PATENT DOCUMENTS

		DOCUMENT	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION YES	TRANSLATION NO
SH		WO96/27798	09/1996	PCT				
SH		WO94/18218	08/1994	PCT				

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent pages, etc.)

SH	PATENT ABSTRACTS OF JAPAN vol. 011, no. 137 (P-572), (05/1987) & JP61 277060 A (JOKO: KK), (12/1986)

*Examiner	[signature]	Date Considered	1/28/05

Examiner: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to application.

Form PTO-FB-A820 (Also PTO-1449)

488552

Notice of References Cited	Application/Control No. 09/743,195	Applicant(s)/Patent Under Reexamination LLOYD ET AL.	
	Examiner Shafiqul Haq	Art Unit 1641	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-5,631,169	05-1997	Lakowicz et al.	436/537
	B	US-5,254,477	10-1993	Walt, David R.	436/172
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Soper et al., Detection and identification of single molecules in solution, 1992, J. Opt. Soc. Am. B, Vol 9, No.10, pages 1761-1769
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Detection and identification of single molecules in solution

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Los Alamos National Laboratory, Los Alamos, New Mexico 87545

Received December 9, 1991; revised manuscript received April 7, 1992

We have extended our recent experiments in the detection of single fluorescent molecules in solution to the exploration of spectroscopy at the single-molecule level. As a first step we have developed a technique that can efficiently distinguish between two species of dye molecules on the basis of differences in their emission spectra. We have also demonstrated that another spectroscopic property, fluorescence lifetime, can be accurately determined at the single-molecule level. Spectroscopic properties can be used to identify fluorescent molecules and to reveal static or slowly varying aspects of the microenvironment of each molecule, thereby yielding information unavailable from bulk studies.

1. INTRODUCTION

The detection of single atoms in the gas phase was first accomplished in 1977,[1] and the detection of single molecules in a solid matrix at low temperatures in 1989.[2] The detection of single chromophore molecules in an aqueous solution presents different problems and was only recently achieved.[3] Because of the many applications of ultrasensitive detection in liquid environments, the sensitivity obtained with fluorescence detection has had a long history of incremental improvements. A new urgency came to the field when it was suggested that DNA may be sequenced at rates many times those of current methods by sequential enzymatic cleavage of an appropriately labeled single DNA strand followed by detection and identification of the individual labeled nucleotides.[4,5]

Hirschfeld[6,7] first reported the detection of a single biological molecule (MW 20,000) that was tagged with 80–100 chromophores. Dovichi *et al.*[8,9] later suggested laser-induced fluorescence as an approach for single-molecule detection in liquids. Recognizing that a greater signal-to-noise ratio could be achieved by reducing the effective sample volume, they set about to reduce the sample volume by using hydrodynamic focusing.[10] Meanwhile improvements were made in the light collection efficiency of flow cytometers,[11] which permitted a detection limit equivalent to 125–150 fluorophores.[12] Using a 0.6-pL sample volume with conventional optics, Nguyen *et al.*[13] demonstrated a detection limit of 800 molecules of Rhodamine 6G. Dovichi and co-workers[14–16] applied ultrasensitive detection techniques to the problem of amino acid analysis by capillary zone electrophoresis. Using B-Phycoerythrin, a large molecule containing the equivalent of 25 Rhodamine 6G (R6G) chromophores, Peck *et al.*[17] observed nonrandom features in the autocorrelation of the signal collected with the passage of many molecules and indicated that they had achieved a detection efficiency of 15% with a false detection rate of 3 errors/s.

Using a new approach in which a rapidly pulsed laser and time-gated detection discriminated against the Raman and other prompt scattering from the solvent, we were able to demonstrate directly the efficient detection and counting of single chromophore molecules in aqueous solution.[3] We also demonstrated that a considerably higher yield of photons per molecule could be obtained by using ethanol as a solvent, owing to better photostability. However, this solvent is incompatible with most biochemical applications, including the DNA-sequencing scheme mentioned above. Subsequently published reports describe the use of continuous laser excitation to provide evidence of sensitivity at the single-molecule level for R6G in ethanol,[18] of detecting as few as 12 molecules of R6G in 12-μm-diameter glycerol droplets,[19] and of detecting small concentrations of R6G in water.[20]

Recently we applied our technique[3] to other fluorescent molecules, including adenine labeled with tetramethylrhodamine isothiocyanate (a fluorophore-labeled nucleotide), and we conducted a study in which the important parameters for single-molecule detection, including photostability, quantum yield, and fluorescence lifetime were measured for several dyes under various solvent conditions.[5]

In the present paper we report the extension of our experiments to the measurement of spectroscopic properties at the single-molecule level. We have developed techniques whereby it is possible to distinguish between two fluorescent species as individual molecules pass through the detector. As a first demonstration of single-molecule spectroscopy we have used differences in emission properties to distinguish between individual molecules of R6G and Texas Red (TR). As another example of single-molecule spectroscopy, we present results of a first experiment on the measurement of the fluorescence lifetimes of individual molecules. We also discuss other spectroscopic properties that may usefully be determined at the single-molecule level.

2. EXPERIMENTAL APPARATUS

The apparatus used in the present experiments is an improved version of that used in our previous study,[3] with modifications to permit excitation and detection at a second pair of wavelengths and to permit the recording of time data on an event-by-event basis. A block diagram is shown in Fig. 1. A mode-locked, frequency-doubled Nd:YAG laser operating at 82 MHz with a pulse width of

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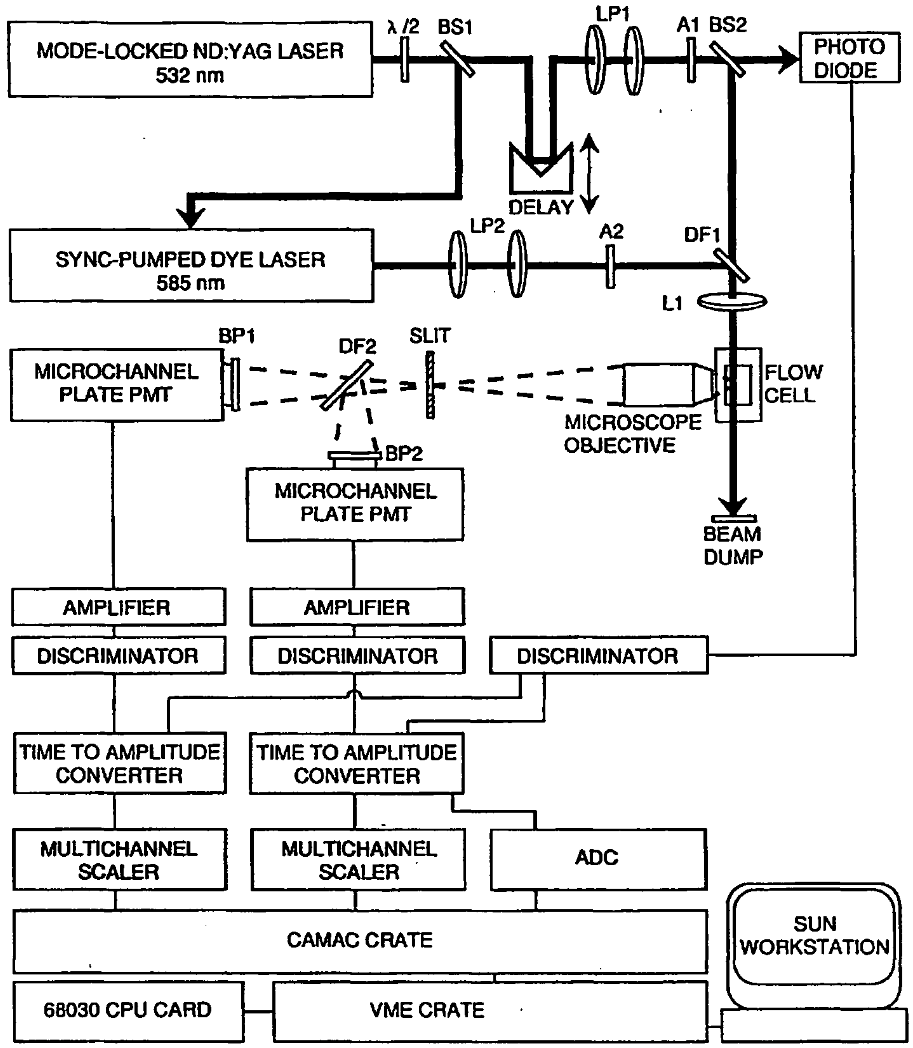

Fig. 1. Block diagram of the single-molecule detection and identification apparatus: λ/2, half-wave plate; BS1, 70%/30% beam splitter; LP1, lens pair for adjustment of size and divergence of the 532-nm beam; BS2, 4%/96% beam splitter for diverting a small percentage of the 532-nm beam to the triggering photodiode; A1, beam attenuator for 532 nm; DF1, dichroic mirror for directing and combining excitation beams; LP2, lens pair used for adjustment of the size and the divergence of the 585-nm beam; A2, beam attenuator for 585 nm; L1, laser focusing lens; DF2, dichroic mirror for separating the fluorescence of the dyes; BP1, BP2, bandpass filters; ADC, analog-to-digital converter; PMT's, photomultipliers.

70 ps was used as an excitation source at 532 nm (green) and also as a pump source for a dye laser. The polarization of the YAG laser was rotated from vertical to horizontal by using a half-wave plate, and beam splitter BS1 was used to deflect approximately 1 W of average laser power to pump the dye laser, while the remaining 400 mW was available as the green excitation source. The green beam was sent through a retroreflector to adjust the timing of the pulse train such that the 532-nm pulses and the pulses from the dye laser arrived at the flow cell at the same time. A fraction of the green laser intensity was split off with beam splitter BS2 and sent to a fast photodiode to provide the start pulse for each time-to-amplitude converter (TAC). The remainder of the green laser intensity was sent through a variable attenuator, which regulated the average power at the flow cell. Dichroic filter DF1 (transmission of 10% at 532 nm, Omega Optical) was used to steer the green light, which was focused into the flow cell by a 20-mm-focal-length laser singlet lens (Melles Griot).

A 585-nm pulse train (yellow) was generated by a locally constructed synchronously pumped dye laser operating with R6G in ethylene glycol. The jet region was enclosed in a Plexiglas box to minimize dye contamination in the laboratory. The tuning element was a three-plate birefringence filter (Coherent Lasers), and the output coupler had a reflectivity of 95%. The cavity length was adjusted to minimize the laser pulse width by observation of the output pulse with a fast photodiode (Antel Model AR-S3). The pulse width was found to be less than the resolution of the photodiode (<50 ps). The yellow light was sent through neutral-density filters to adjust the average power at the flow cell and through dichroic filter DF1 (transmission of 95% at 585 nm) and then was focused into the cell with the same lens used to focus the green light. Lens pairs were used in the green and the yellow beam paths to adjust the size and the divergence such that both beams focused at the same plane and had the same beam-waist diameter. The size of the beam waist was determined by imaging Rayleigh and Raman scattering from the solvent

onto a linear diode array (EG&G, Model RL0512). The beam waist for both the green and the yellow laser beams was 6.0 μm (1/*e* intensity radius). The two beams were aligned to be collinear within the flow cell and were positioned approximately 400 μm from the wall of the flow cell, whose internal dimensions were 1 cm $\times$ 0.5 cm.

The dilute dye solution was delivered to the flow cell from a bottle pressurized with helium or air. The pressurized delivery system permits O_2 and other atmospheric gases to be purged from the solution if desired. The polyethylene tubing connecting the solvent bottle to the flow cell was enclosed in thick-walled Tygon tubing in which N_2 flowed to exclude O_2, which might otherwise have diffused through the polyethylene. The ultradilute dye solutions were made by serial dilution from stock solutions of Kodak-supplied dye powders dissolved in CH_3OH.

Fluorescent light from dye molecules passing through the laser beams was collected by a 40×, 0.65-N.A. (Zeiss) microscope objective and imaged onto a 400-μm-aperture vertical slit, producing a viewing distance along the propagation axis of the laser beam of 10 μm. The resulting detection volume was 1.1 pL. The probability of a dye molecule's occupying the detection volume is 0.007 at a concentration of 10^{-14} M. The fluorescent light was separated and directed onto separate photodetectors by dichroic filter DF2 (Omega Optical). The fluorescence of R6G and TR was isolated with eight-cavity bandpass filters BP1 (cavity wavelength CWL = 555 nm, FWHM = 30 nm for R6G) and BP2 (CWL = 605 nm, FWHM = 30 nm for TR; Omega Optical). The transmission spectra of the bandpass filters and filter DF2 and the fluorescence emission spectra of R6G and TR are shown in Fig. 2. Integration of the bandpass region of the fluorescence isolation filters over the emission spectra of the dyes indicated that approximately 60% of the fluorescence of the dyes was transmited through the filters. Detector 1 for R6G and detector 2 for TR were 12-μm proximity-focused microchannel plate photomultipliers (Hamamatsu Model R1564U) cooled to 0°C with bialkali and extended-red multialkali photocathodes, respectively. The outputs were amplified (Hewlett Packard, Model 8447F) and sent to separate constant-fraction discriminators (Tennelec Model TC454) and TAC's (Tennelec Model TC863). Pulses from the constant-fraction discriminators were used both to open the corresponding TAC start-signal gate so that the photodiode signal could pass, thereby initiating a conversion cycle and, after an appropriate timing delay, to provide the stop pulse for the TAC. Typical time spectra from each detection channel are shown in Fig. 3. The FWHM of the prompt peaks was approximately 150 ps for both detectors. The position and the width of the time windows in which fluorescent photons were accepted were optimized by observing the magnitude of the photon burst from each dye and by minimizing the background with a real-time display.

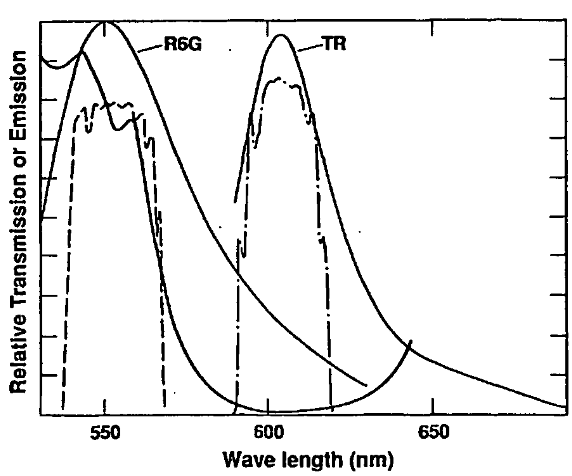

Fig. 2. Fluorescence emission spectra of R6G and TR (thinner solid curves), the transmission spectra of the fluorescence isolation dichroic filter DF2 (heavy solid curve), and the fluorescence bandpass filters (dashed and dotted–dashed curves).

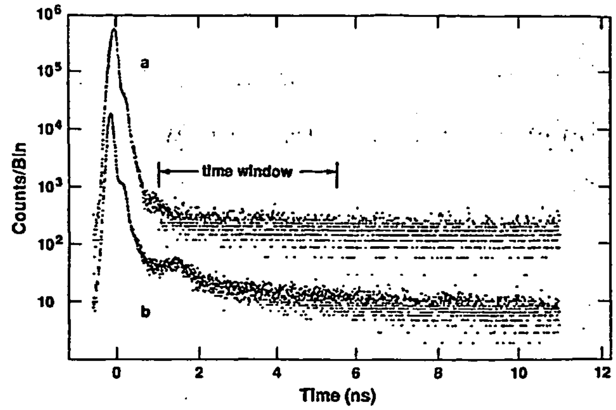

Fig. 3. Time spectra for CH_3OH from a, detector 1, and b, detector 2. Spectrum a was shifted upward for clarity. The time window in which the fluorescent photons were recorded by the multichannel scalers is shown. These windows contained approximately 60% of the photons available from the entire decay. The small bump near 1.5 ns is caused by reflected light.

The real-time display was implemented by computing the weighted-quadratic-sum algorithm (see Section 3) online with a Sun workstation. A VME-bus color monitor driven by the Sun workstation provides a chart-recorder-style display of the amplitude of the bursts that occur as individual dye molecules pass through the flow cell. Counts occurring within the time windows were also sent to computer automated measurement and control (CAMAC) multichannel scalers (Joerger Model S3) for off-line analysis. The CAMAC bus was interfaced to the VME bus (via Creative Electronic Systems Model CDB-8210) and hence to the Sun workstation (via Performance Technology model PT-VME 901).

For the lifetime measurements the TAC output was digitized by a 10-bit CAMAC analog-to-digital converter (ADC, LeCroy 3511), which recorded the time difference between laser pulse and photon arrival with a resolution of approximately 10 ps. To identify photon bursts, one needs the arrival time of each photon. This was provided by the internal clock of the VME CPU card (Motorola MVME 147S) that managed the event-by-event data collection for the lifetime measurements. Real-time software on the CPU card ran under VxWorks and communicated with the Sun workstation via Ethernet.

3. RESULTS OF EXPERIMENTS

Our initial experiments used pure (unmixed) solutions of R6G and TR to verify that the separate detection channels were aligned and operating correctly and that indi-

vidual molecules of each separate dye could be efficiently detected. This having been accomplished, the next step was to evaluate the cross talk for each dye as seen by the opposite detection channel. For these tests CH_3OH was used as the solvent, since with alcohol an average of $\sim 10^6$ photons per molecule are available before photobleaching, while in aqueous media the yield is only 10,000–30,000 photons. Thus alcohol can provide a more sensitive indication of cross talk between detection channels.

Autocorrelation analysis of the instantaneous photon-counting rate has been shown[17] to be a sensitive indicator of the nonrandom signal produced as molecules pass through the laser beam. Accordingly we have used this method to investigate cross talk. For a data set $d(t)$ consisting of N consecutive values of the number of photons detected in a specified time interval, the digital autocorrelation function is given by

$$A(\tau) = \sum_{t=0}^{N-1} d(t)d(t+\tau). \tag{1}$$

The function $A(\tau)$ is plotted in Fig. 4 for data streams obtained with R6G and TR separately and with CH_3OH alone. For detector 1 (the R6G detection channel) the presence of TR results in no autocorrelation above that observed with CH_3OH alone, although a slight increase in the average counting rate causes an upward shift in the entire autocorrelation plot. The small increase in the autocorrelation function at short time delays that is observed in both data sets results from the detection of fluorescence impurities in the solvent. As expected, when R6G is added, the autocorrelation peak in the R6G detection channel is greatly increased. Corresponding results are observed for detector 2 (the TR detection channel), which indicates that both detection channels have little or no sensitivity to the opposite dyes.

Next a mixed dye solution of 10^{-14} M R6G and 10^{-14} M TR in CH_3OH was used to test our ability simultaneously to detect and identify individual dye molecules of two types in a mixture by means of their specific emission properties. At this concentration approximately

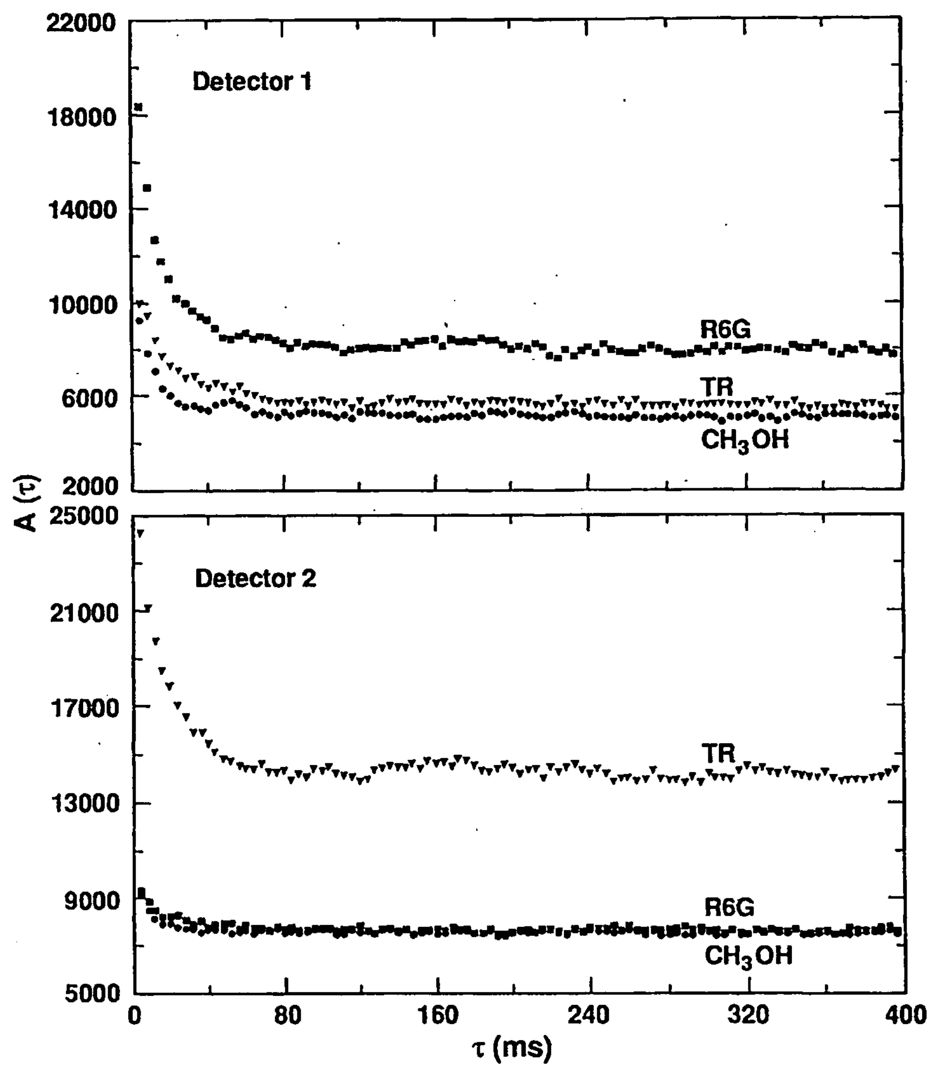

Fig. 4. Autocorrelation functions for CH_3OH, 1×10^{-14} M R6G and 1×10^{-14} M TR solutions for detectors 1 and 2. The average laser power used in these experiments was 30 mW each for 532- and 585-nm excitation. The data were collected in 1-ms time widths (204,800 total data points) and then binned by 4 before analysis. The $A(0)$ points are off scale.

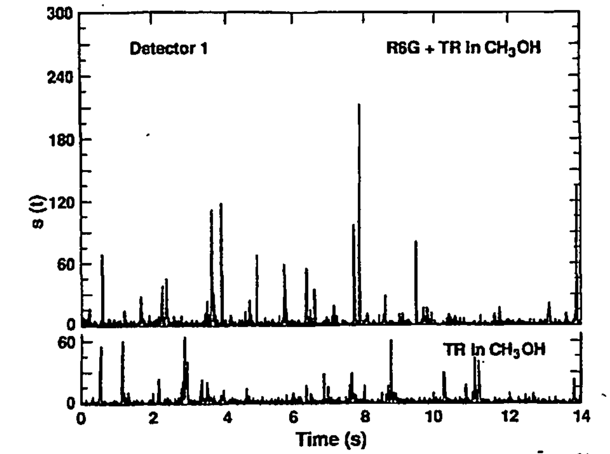

Fig. 5. Weighted-quadratic-sum plots for a mixed 1×10^{-14} M methanol solution of TR and R6G (top) and TR alone (bottom) from detector 1, the R6G channel. At 10^{-14} M approximately one dye molecule enters the detection volume every 4 s.

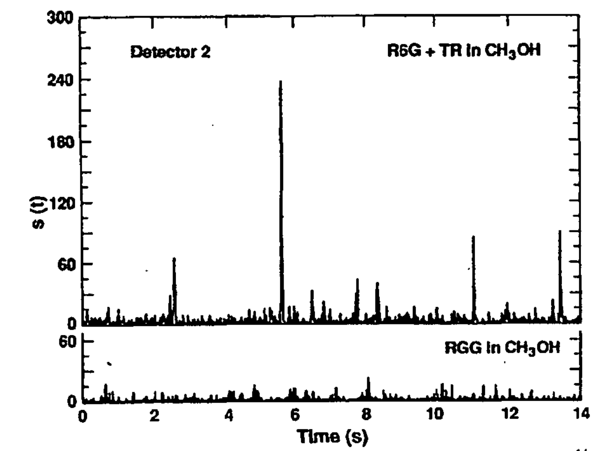

Fig. 6. Weighted-quadratic-sum plots for a mixed 1×10^{-14} M methanol solution of TR and R6G (top) and R6G alone (bottom) from detector 2, the TR channel.

one molecule of each type enters the detection volume every 4 s. Because the transit time of a molecule through the volume is only ~40 ms, there is a negligible chance that two or more molecules are simultaneously present. A data stream, which consists of the number of photons detected in successive 2-ms time bins, was recorded from each detection channel. Each data stream was processed with the weighted-quadratic-sum filter $S(t)$ that we have used in previous single-molecule-detection experiments[3]:

$$S(t) = \sum_{\tau=0}^{k-1} w(\tau)d(t + \tau)^2. \tag{2}$$

The range k covers a time interval that is approximately equal to the lesser of the molecular passage or the mean photobleaching time; in the present study the values $k = 9$ and $k = 13$ were used for water and methanol solvents, respectively. The weights $w(\tau)$ are chosen best to distinguish the signal from passing molecules from random fluctuations in the background. Typically the fluorescence signal increases slowly as the molecule approaches the laser beam, followed by an abrupt cessation when photobleaching occurs. We therefore choose $w(\tau)$ as an asymmetric triangular ramp: $w(\tau) = (\tau + 1)/k$ for $\tau = 0$ to $k - 1$ and $w(\tau) = 0$ otherwise. This filtering operation helps to reveal the bursts of photons that signify a passing molecule.

The filtered photon-burst data from the mixed dye solution are shown in the top sections of Figs. 5 and 6 for the two detection channels. Large-amplitude bursts that correspond to individual molecules are clearly evident in both plots. From the previous autocorrelation cross-talk measurements, we can expect detector 1 to respond primarily to R6G, and detector 2 to TR. This is easily confirmed by using separate R6G and TR solutions in place of the mixed solution. Filtered data streams for each dye alone are shown in the lower sections of Figs. 5 and 6. Because detector 2 (the TR channel) shows little background from R6G, single molecules of TR can be efficiently detected even in the presence of R6G molecules. Detector 1 (the R6G channel) shows occasional medium-height peaks with the TR solution. We believe that these originate primarily from impurities in the CH_3OH, because they are present even with the solvent alone.

Evaluating the error rate and detection efficiency for the detector requires that the threshold discriminator level be set at some value of $S(t)$. One then asks how often the background signal exceeds that level when the sought dye is absent and what fraction of sought dye molecules produce signals above that level. To keep the number of false-positive errors from a blank solution below an arbitrarily chosen value of 0.01 errors/s, the discriminator levels were set at 86 for detector 1 and 65 for detector 2. When the sought dye was present at the concentration of 10^{-14} M, the discriminator levels yielded averages of 35 and 40 events during 205-s runs for detectors 1 and 2, respectively.

The number of dye molecules that pass through the detection volume (defined at the $1/e$ intensity contour) is given by $N = c\sigma vT$, where c is the concentration of dye molecules in the solvent, σ is the cross-sectional area defined by the midplane $1/e$ laser-beam intensity contour and the spatial filter slits, v is the flow velocity, and T is the duration of the experiment.

The linear flow velocity cannot be readily deduced from the volumetric flow rate, since the laser beams were focused near the wall of the rectangular cell in a region of reduced flow velocity. (This focus was mandated by the limited working distance of the microscope objective). Instead the flow velocity was calculated from autocorrelation data for R6G and TR by fitting the peak near the origin to a Gaussian. If a dye molecule passes through a Gaussian beam without diffusion or photodegradation, the photon burst will on average be Gaussian in shape, and the autocorrelation function will exhibit a Gaussian peak with a width that is $\sqrt{2}$ times the width of the burst. Diffusion and photodegradation will modify this width, so we estimated their contributions to the autocorrelation by combined analytical and Monte Carlo calculations that incorporated the photophysical and diffusion coefficients of the dyes and the geometry of the experiment. The photodegradation effect proved to be negligible (fewer than 10% of the molecules photodegrade), whereas diffusion reduced the width of the autocorrelation peak by ~35%. After correction the flow velocity was determined to be 290 μm/s, indicating that approximately 45 mole-

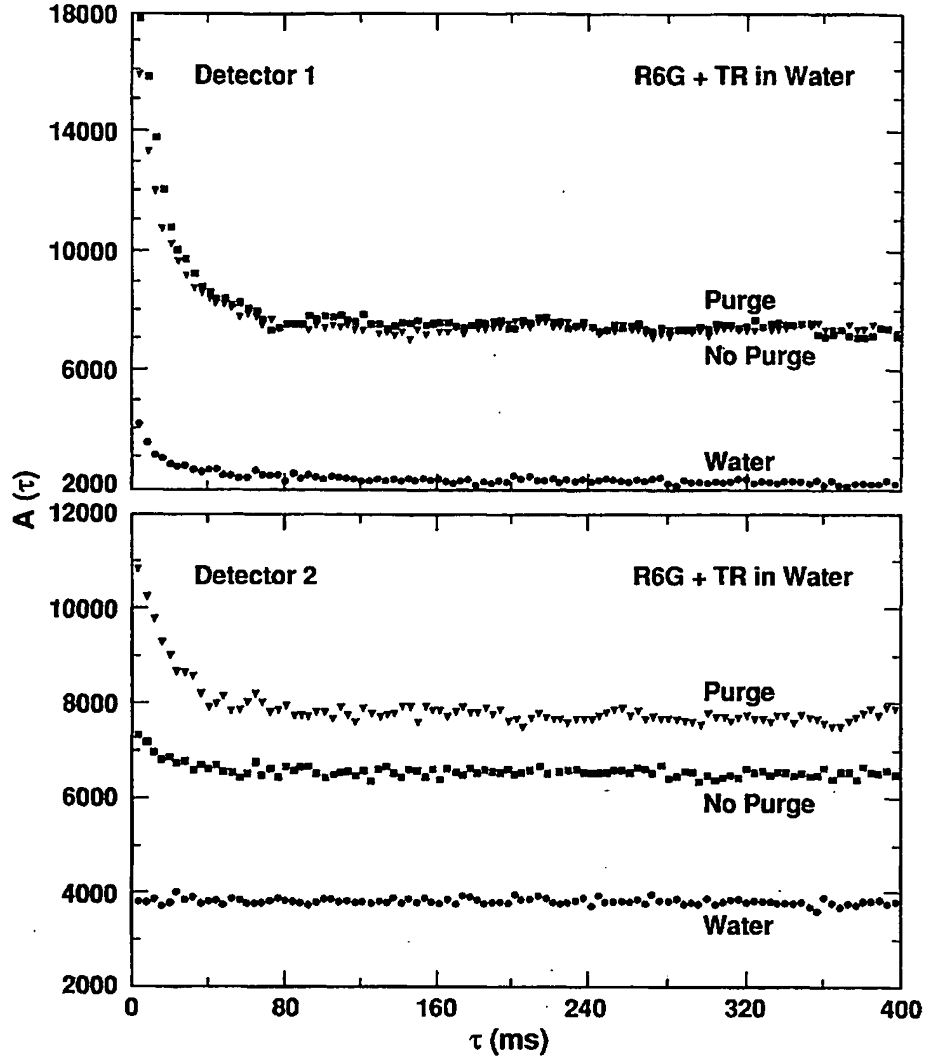

Fig. 7. Autocorrelation plots for water and for mixed 5×10^{-14} M R6G and TR in water, either O_2 saturated (no purge) or deoxygenated (purged), for both detectors. In both plots the purged data are represented by triangles.

cules pass through the $1/e$ volume of the detector during a typical 205-s experimental run.

The efficiency for detection, expressed as the ratio of detected molecules to the number that enter the probe volume, can now be calculated. For R6G with a discriminator level of 86, the efficiency is 78% with an error rate of 0.01 error/s, whereas for TR the efficiency is 90% with the discriminator level set at 65 and an error rate of <0.01 error/s. Note that it is possible to detect more molecules than those that pass through the central $1/e$ portion of the detection volume if molecules on the fringes of the laser beam are also detected; this can lead to an efficiency of greater than 100% by the present definition. The uncertainty of the efficiency values given above and elsewhere in this paper is not easy to assess. The primary uncertainty is probably in the molarity of the dye solutions at the low concentrations required in these experiments. These dilute solutions were made by successive dilutions from a weight-based stock solution, and the final concentration was computed without considering possible adsorption of dye molecules to glass surfaces during the dilutions or to the polyethylene tubing that connects the supply reservoir to the detection cell. We believe that if the dye concentration values were in error, the actual concentrations would be lower than we have assumed, which would lead us to underestimate the efficiency of the detector.

Because an aqueous environment is much more suitable for many biochemical applications of single-molecule spectroscopy, we repeated the experiments with water as the solvent. Identification of individual molecules of R6G and TR in aqueous solutions is more difficult than in alcohol because the quantum efficiency and photostability of both dyes are lower in water. In Fig. 7 we show autocorrelation data for mixed 5×10^{-14} M TR and R6G in water for solutions with and without helium purging to remove O_2. O_2 removal has been shown to diminish photobleaching in fluorescence microscopy.[21] As can be seen, a strong autocorrelation peak occurs for R6G (detector 1) with or without purging, whereas for TR (detector 2) a strong peak only occurs when O_2 is purged. Even with deoxygenation, in aqueous solutions TR evidently produces burst amplitudes much smaller than R6G.

Figures 8 and 9 show filtered data streams like those of

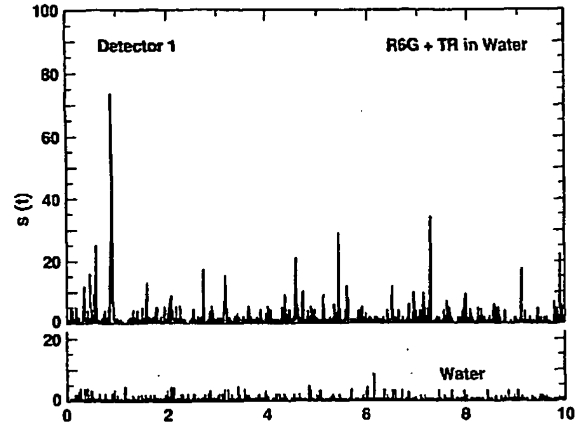

Fig. 8. Weighted-quadratic-sum plots for a mixed 5×10^{-14} M O_2-purged water solution of R6G and TR (top) and purged water alone (bottom) from detector 1. For the water data the value of $k = 9$ was used in the weighted-quadratic-sum filter procedure. At 5×10^{-14} M approximately one dye molecule passes through the detector every second.

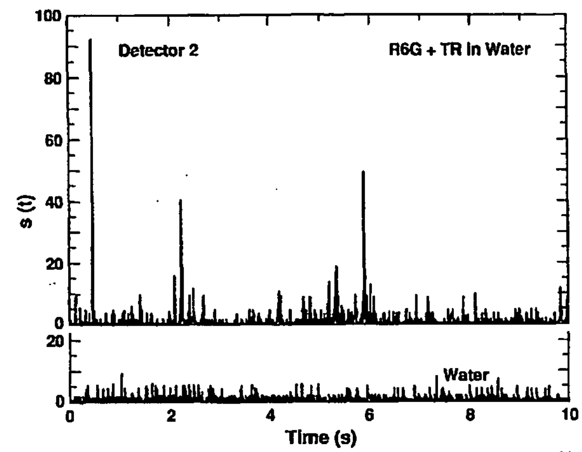

Fig. 9. Weighted-quadratic-sum plots for a mixed 5×10^{-14} M, O_2-purged water solution of R6G and TR (top) and purged water alone (bottom) from detector 2. See the caption of Fig. 8.

Figs. 5 and 6, except that O_2-purged water is the solvent in place of methanol, and we show background data obtained from pure water, since cross talk was shown to be negligible in the more-sensitive methanol experiments. The filtered data streams from both detection channels exhibit peaks in response to a mixed aqueous dye solution (5×10^{-14} M in each dye), indicating that individual molecules are being detected. The streams shown were taken at the same time, but there is no evident correlation between the time peaks that occur in the two channels, again indicating the absence of cross talk. In water typical bursts from single dye molecules contained 10–20 detected photoelectrons, which is several times fewer than we observed in methanol. We estimated the detection and identification efficiency as before by arbitrarily selecting $S(t)$ thresholds that keep the false positives below 0.01 error/s. The resulting efficiency values for R6G (detector 1) are 87% without O_2 purging and 79% with purging. For TR (detector 2) the efficiencies were 24% without purging and 54% with purging. Interestingly, deoxygenation improves the detection efficiency for TR while reducing it for R6G. We observed that the effect of O_2 purging varied with the length of time that the solution was purged before the measurements began. Prepurging times greater than the 30-min prepurge used when the efficiency values given above were obtained further reduced the detection efficiency for R6G.

Other spectroscopic properties can be studied or measured at the single-molecule level. For example, we show in Figs. 10 and 11 the results of an experiment that indicates that the fluorescence lifetime of an individual molecule can be measured with reasonable precision. For this experiment we used TR in methanol to minimize photodegradation and thus to record a large number of photons from each molecule. The dye concentration (2.5×10^{-14} M) and flow velocity were such that during runs lasting several minutes only a few dozen dye molecules passed through the detection volume. For each photon arriving at the detector, a pair of time values was recorded: (1) the time of photon arrival from a continuously running (time-of-day) clock and (2) the time difference (ΔT) between laser pulse and photon arrival. The solvent flow rate was sufficiently low (<50 μm/s) that diffusion largely determined the residence time of the molecules in the detection volume. Burst profiles often exhibited multiple peaks (see Fig. 10) as a molecule wandered into, out of, and back into the laser beam. Between bursts the photon detection rate was low and corresponded to the background rate. Because the bursts were few and well separated, they were easy to identify. The individual bursts, which represent the repeated excitation of a single dye molecule, often contained a few hundred detected photons.

We selected an individual burst and examined the ΔT values for each of the photons that made up that burst (see Fig. 11). These time values are single observations of the fluorescence lifetime of an individual molecule. Taken together these few hundred values yield a reasonably accurate measurement of the fluorescent lifetime of that molecule, which can be used to identify the molecular species that produced the burst. When a single decay expo-

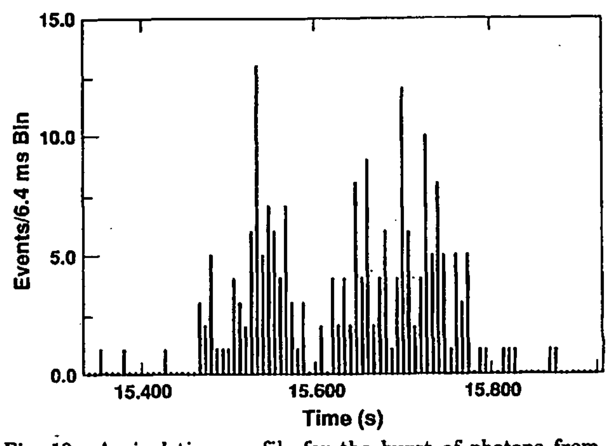

Fig. 10. Arrival time profile for the burst of photons from a single molecule. The time scale on the horizontal axis has an arbitrary origin; this particular burst occurred ~15.5 s after the start of the experiment. An irregular multiple-peaked profile such as this is often seen when a molecule diffuses into, out of, and back into the detection volume. A total of 215 photons was detected from this particular burst, of which fewer than 10 were background.

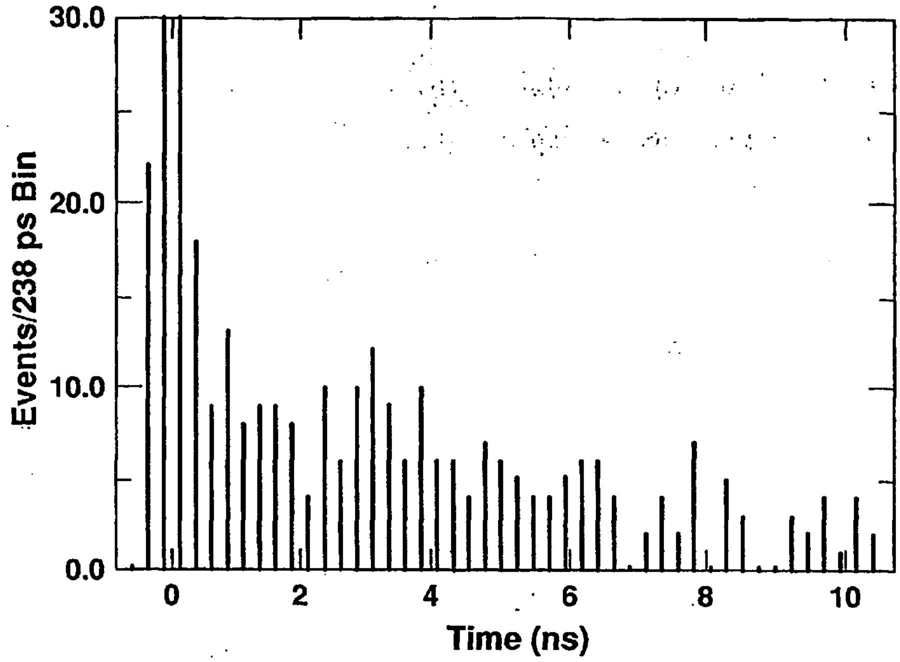

Fig. 11. Estimating the lifetime of a single molecule. The decay time (difference between time of the laser pulse and time of photon arrival) associated with each photon detected during the burst shown in Fig. 10 was histogrammed to produce the noisy exponential decay profile shown. From this data set the maximum-likelihood estimate of the lifetime of this particular molecule was 4.5 ± 0.3 ns, in agreement with the value 4.17 ± 0.01 ns determined by measurements on bulk solutions (see text). Lifetimes computed for other individual bursts were also clustered near the bulk value. The two bins near $t = 0$ are far off scale because they include prompt Raman-scattered photons. (Only delayed events were included in the profile shown in Fig. 10.)

nential is assumed and the number of background events during the brief duration of the burst is negligible, as it is for the data shown in Fig. 10, the maximum-likelihood estimate of the fluorescence lifetime takes a particularly simple form—the mean of the individual observations. For a burst of size N, the relative error of this lifetime estimate is $N^{-1/2}$. Thus, from an observed burst of 200 photons, the lifetime can be determined with an accuracy of 7%, which is sufficient for distinguishing many species of dyes.

For the particular burst shown in Figs. 10 and 11 we computed the lifetime to be 4.5 ± 0.3 ns, in agreement with the value 4.17 ± 0.01 ns determined by measurements for a bulk solution of TR in methanol. Lifetimes computed for other individual bursts were also clustered near the bulk value. When inferring the lifetime from the mean of the observed ΔT values, we have corrected for the fact that the laser repetition rate prevents us from recording values of ΔT greater than ~10 ns. In this connection we note that a constant background will not strongly bias the lifetime derived from the mean of the ΔT values if ΔT values spanning a range equal to twice the lifetime are used to compute the mean. Even though the present background was quite small, this condition was approximately satisfied in our experiments. Since the origin for ΔT need not be the time of the excitation pulse, we took $\Delta T = 0$ at 0.7 ns after the excitation pulse to eliminate scattered photons. A rigorous treatment of the complete decay profile, including prompt photons and background, by maximum-likelihood methods is the topic of a separate study.[22]

DISCUSSION AND CONCLUSIONS

As examples of spectroscopy at the level of individual molecules, we have demonstrated that differences in emission spectra, and perhaps in fluorescence lifetime, can be used to identify single dye molecules in a mixture. Fluorescence quantum yield is another spectroscopic property that could readily be measured at the single-molecule level to provide a means of distinguishing among molecular species. Observation of the photon emission rate as individual molecules pass through a laser beam of known intensity can provide an absolute or relative determination of quantum yield (strictly, the product of the absorption coefficient and the quantum yield). The present experimental arrangement did not allow us to make such measurements, since different molecules experience different light intensities because of the Gaussian beam profile. Similarly, the photodestruction lifetime could be measured at the single-molecule level by observing the duration of the burst by techniques similar to those used for Fig. 10. However, diffusion and other factors may complicate the interpretation.

With proper experimental design two or more independent spectroscopic properties, e.g., lifetime and emission spectra, could be observed simultaneously for each passing molecule. Knowledge of two or more parameters would localize each molecule in a multidimensional parameter space and would be useful in cases in which the value of a single parameter would be insufficient to permit a definitive identification.

We have discussed single-molecule spectroscopy primar-

ily from the point of view of identification of molecular species, as when a dye is attached to a nonfluorescent molecule as an identification tag. However, the capability of performing spectroscopic measurements on individual fluorescent molecules will have a range of other applications in chemical and biochemical studies. To the extent that spectroscopic properties are modified by the immediate environment of a molecule, information about that microenvironment can be obtained. Data collected by observing individual molecules would reveal features that are not evident in the average behavior of a group. For example, measurement of the average quantum yield of a group of chromophores of which one in 50 is bound to a quencher would not reveal the underlying statistics of the quenching, whereas this information would be readily accessible with single-molecule spectroscopy. Phenomena that occur in microscopic-sized samples and that involve only a few molecules can be studied. When viewed in this light, the present spectroscopic techniques provide tools for studying chemical and biological interactions that are inaccessible to macroscopic methods.

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